Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression

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Human plasma membrane-associated sialidase (Neu3) is unique in specifically hydrolyzing gangliosides, thought to participate in cell differentiation and transmembrane signaling, thereby playing crucial roles in the regulation of cell surface functions. We have discovered levels of mRNA for this sialidase to be increased in restricted cases of human colon cancer by 3- to 100-fold compared with adjacent nontumor mucosa ($n = 32$), associated with significant elevation in sialidase activity in tumors ($n = 50$). *In situ* hybridization showed the sialidase expression in epithelial elements of adenocarcinomas. In cultured human colon cancer cells, the sialidase level was down-regulated in the process of differentiation and apoptosis induced by sodium butyrate, whereas lysosomal sialidase (Neu1) was up-regulated. Transfection of the sialidase gene into colon cancer cells inhibited apoptosis and was accompanied by increased Bcl-2 and decreased caspase expression. Colon cancer exhibited a marked accumulation of lactosylceramide, a possible sialidase product, and addition of the glycolipid to the culture reduced apoptotic cells during sodium butyrate treatment. These results indicate that high expression of the sialidase in cancer cells leads to protection against programmed cell death, probably modulation of gangliosides. This finding provides a possible sialidase target for diagnosis and therapy of colon cancer.

Aberrant glycosylation is known to be a common feature of cancer cells. In particular, alteration of sialic acids, generally found in the nonreducing terminus of most glycoproteins and glycolipids, is associated with cancer cell behavior, such as invasiveness and metastasis (1–5). A general increase in sialylation is often found in cell surface glycoproteins of malignant cells (6), and altered sialylation in glycolipids is also observed as a ubiquitous phenotype, leading to the appearance of tumor-associated antigens, aberrant adhesion, and blocking of transmembrane signaling (7, 8).

To define the significance and molecular mechanisms of aberrant sialylation in cancer, we have focused attention on mammalian sialidase, a key enzyme in catabolism of glycoproteins and glycolipids that cleaves sialic acid residues from glycoconjugates. Endogenous sialidases of mammalian cells have been classified into three or four types differing in subcellular localization and enzymatic properties, and this multiple nature suggests that each form may play a unique role depending on its properties. Of the three types so far cloned (Neu1, Neu2, and Neu3), we were successful in cloning two types of sialidase cDNA, located mainly in cytosol (Neu2) (9) and plasma membranes (Neu3) (10, 11). Human Neu3 is a unique glycosidase in its subcellular localization and strict substrate preference to gangliosides (12–14). Unlike lysosomes, the membranes do not contain a set of glycosidases to degrade glycoproteins and glycolipids, thereby Neu3 probably plays crucial roles in the regulation of cell surface functions other than catabolism of glycoconjugates. Previous studies on a ganglioside sialidase in murine malignant cells (15, 16) have suggested that altered expression of the sialidases is likely to be related to malignant transformation, although it was not identified to be Neu3 at that time. In this article, we provide evidence that human Neu3 is indeed significantly up-regulated in colon cancer. Moreover, Neu3 may act to modulate apoptosis and cell differentiation in the cancer cells.

Materials and Methods

Patient Samples. Surgical specimens were obtained from 50 colon cancer patients (aged 24–83 years, mean 66.1 ± 10.7 years), 24 males and 26 females, who underwent resection of their tumors at Miyagi Prefectural Cancer Center. The histological differentiation of the tumors was well (12 patients), moderate (35 patients), and mucinous (three patients), and pathological stages were for pTNM classification: stage 1 for two patients, stage 2 for 24, stage 3 for 16, and stage 4 for eight. Samples were taken from nonnecrotic areas of tumors and adjacent nontumor tissue and immediately frozen on dry ice. Informed consent was obtained from each patient to allow the use of portions of tissue for research purposes, and the study was approved by the Committee on Human Rights in Research at Miyagi Prefectural Cancer Center.

Tissue Culture. Human colon cancer cell lines used in this study were HCT-15 and Colo-205 (Cancer Cell Repository, Tohoku University, Sendai, Japan), DLD-1 (Health Science Research Resources Bank, Osaka) and HCT-116 (American Type Culture Collection). The cells were maintained in DMEM containing 10% FBS and antibiotics.

Neu3 Transfection. To obtain stable transfectants, an ecdyson-inducible expression kit (Invitrogen) was used as recommended by the manufacturer. The ecdyson-inducible Neu3 expression vector (pIND-HmSD) was constructed by subcloning the ORF (1.2 kb) of human Neu3 cDNA (11) into a pIND vector and cotransfected with a pVgRXR vector into HCT-116 cells with Lipofectamine (GIBCO/BRL). Positive clones were selected under G418 (800 μg/ml) and zeocin (200 μg/ml), and the cells were treated with ponasterone A (5 μM) for 24 h to induce Neu3. For transient transfection, a Neu3 expression vector was constructed by subcloning the ORF of human Neu3 cDNA into pCEP4 (Invitrogen), introduced into HCT-15 and DLD-1 cells with Lipofectamine, and harvested after 48 h.

Competitive Reverse Transcription–PCR (RT-PCR) Analysis. Human Neu3 mRNA level was evaluated by quantitative RT-PCR with a cDNA competitor prepared by *NcoI* digestion of Bluescript vectors containing ORF of human Neu3. Primers were sense (5′-GACAGAGGGATTACCTACCGGATC-3′, nucleotides 55–78 from the start codon) and antisense (5′-GAGCCATGATTCT-

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Abbreviations: NaBT, sodium butyrate; Lac-cer, lactosylceramide; RT-PCR, reverse transcription–PCR.

See commentary on page 10231.

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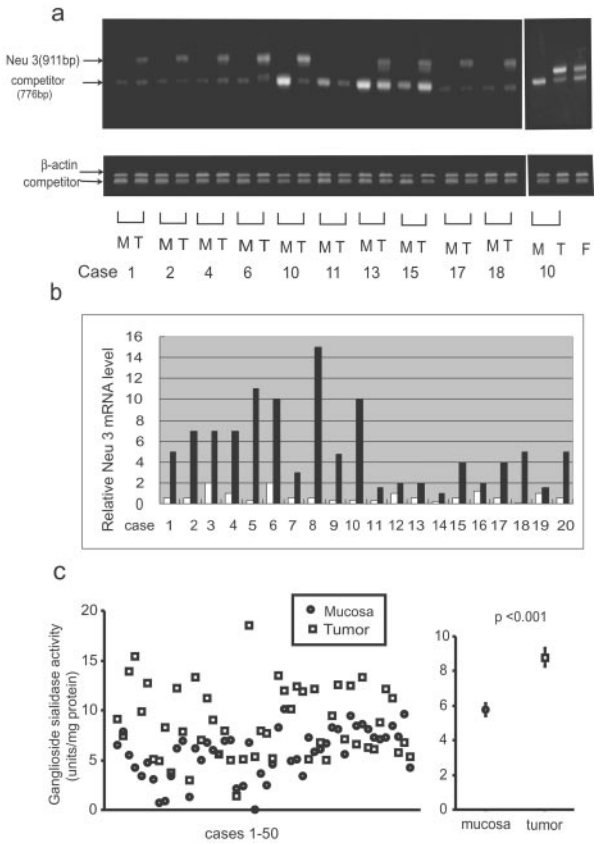

Fig. 1. Neu3 expression in colon cancers and noncancerous mucosa. Representative results for quantitative RT-PCR patterns (*a*) and relative Neu3 mRNA levels (*b*) of tumor tissues from 10 and 20 patients, respectively, are compared with those from adjacent nontumorous tissues. Neu3 mRNA level was measured by RT-PCR by using 5 fg of Neu3 competitor DNA (*a*), after normalization for sample variation by β-actin expression (*a Lower*). M, mucosa; T, tumor; F, fetal colon. (*b*) Precise quantification based on the first PCR was then performed by using several amounts of Neu3 competitor. Open and closed columns indicate the values for noncancerous mucosa and colon cancer, respectively. (*c*) The sialidase activity was assayed for Neu3 in 50 pairs of tumor and adjacent nontumor tissues.

GACGGTGTT-3′, nucleotides 966–987). First-strand cDNAs were synthesized from total RNA of the tissues and cells by RT and used as templates for PCR as described (11). To normalize for sample variation, β-actin expression was measured as an internal control by using a β-actin competitor, prepared by digestion of the cDNA (17) (nucleotides 25–1075) with *Bst*EII and *Msc*I. For colon cancer cells, Neu1 mRNA levels were also estimated by using a competitor prepared by digestion of the cDNA (nucleotides 1–1249) (18–20) with *Bbs*I. Densitometric analyses of PCR products were performed with National Institutes of Health IMAGE.

***In Situ* Hybridization.** Sense and antisense probes were generated by using digoxigenin (DIG)-labeled UTP (Roche Molecular Biochemicals) and a Bluescript plasmid containing human Neu3 cDNA fragment (nucleotides 1–928) and fragmented to approximately 300 bp by alkaline treatment. Frozen tissue sections were fixed and hybridized with the probes as described (21). Positive signals were detected with anti-DIG–alkaline phosphatase conjugate and nitroblue tetrazolium/X-phosphate.

Sialidase Activity Assay. Cell homogenates and the particulate fractions of tissue homogenates were prepared as described (10, 12) and used for Neu3 sialidase assay at pH 4.6 with mixed gangliosides (Sigma) as a substrate in the presence of Triton X-100. The released

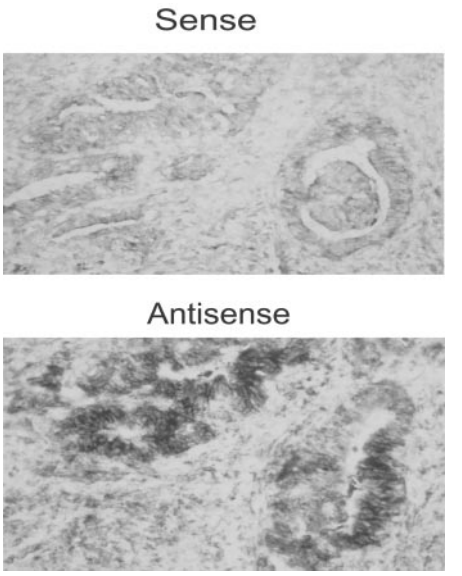

Fig. 2. *In situ* hybridization analysis of Neu3 in a colon cancer. Frozen sections of colon cancer tissue were hybridized with digoxigenin-labeled Neu3 antisense and sense RNAs. All photographs were taken at 40 × 10 magnification.

sialic acids were determined by the thiobarbituric acid method after passing through an AG1X-2 minicolumn. For Neu1 sialidase, the enzyme fraction was incubated at pH 4.6 with synthetic substrate 4-methylumbellyferyl-neuraminic acid, and 4-methylumbellyferone released was determined fluorometrically (22). One unit was defined as the amount of enzyme that cleaved 1 nmol of sialic acid/h.

Alkaline Phosphatase Activity Assay. Cell homogenates were assayed with 2-amino-2-methyl-1-propanol buffer and *p*-nitrophenyl phosphate substrate (Sigma). After termination of the reaction with 0.05 M NaOH, the release of *p*-nitrophenol was determined at 410 nm. Enzyme activity was expressed in Sigma units/0.1 mg protein.

Analysis of Bcl-2 and Caspase Expression by Western Blot. Cell homogenates (35 μg protein) were electrophoresed on a 12% SDS/PAGE gel, and the proteins transferred to poly(vinylidene difluoride) membranes were Western-blotted with specific antibody by using an ECL system (Amersham Pharmacia). Antibodies to the following proteins were purchased: Bcl-2 (Bcl-2–100, Sigma), β-tubulin (AC-15, Sigma), cleaved caspase-3 (Asp-175, New England Biolabs), and cleaved caspase-9 (Asp-330, Cell Signaling Technology).

Apoptosis Assay. Apoptosis analyses were performed by annexin V staining and terminal deoxynucleotidyltransferase-mediated dUTP end labeling (Annexin-V Fluos staining kit and *in situ* cell death detection kit, respectively, Roche Molecular Biochemicals) as recommended by the supplier.

TLC. Glycolipids were extracted from cells (10^7) and tissues (0.4–0.5 g each) as described (21), and the crude lipid extract was applied to a DEAE-Sephadex A25 minicolumn (0.5 × 1.5 cm). Neutral glycolipids were eluted with chloroform/methanol/water (30:60:8, vol/vol/vol) and gangliosides with the same solvent containing 1 M CH_3COONa. Neutral fractions were then treated by alkaline hydrolysis. A tenth amount of each sample was chromatographed on high-performance TLC plates (Baker). Neolactotetraosylceramide, Le^x ($III^3FucnLc_4$-cer), and Le^a (III^4FucLc_4-cer) were prepared by incubating their respective sialyl compounds (Wako

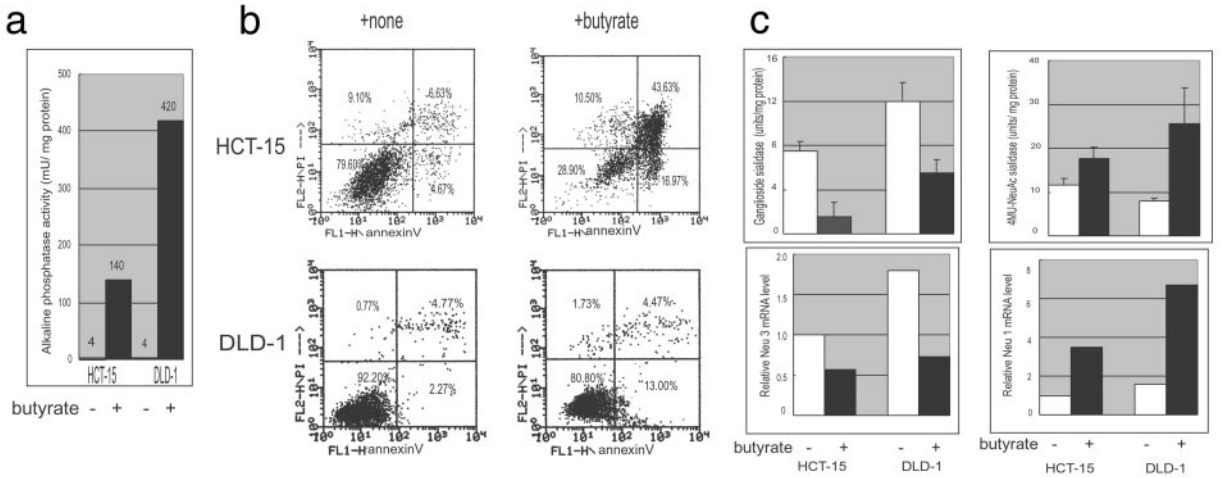

Fig. 3. Neu3 and Neu1 sialidase expression during NaBT–induced differentiation and apoptosis of human colon cancer cells. Alkaline phosphatase activity was measured by using cell homogenates (*a*), and extent of apoptosis was assessed by annexin V staining (*b*) after 50 h of treatment in culture. Propidium iodide (*y* axis) and annexin V (*x* axis) were used as dual parameters. (*c*) The sialidase activities for Neu3 and Neu1 (*Upper*) were assayed with gangliosides and 4-methylumbellyferyl-neuraminic acid as substrates, respectively, and the mRNA levels were quantified by competitive RT-PCR (*Lower*).

Biochemicals, Osaka) with *Arthrobacter ureafacience* sialidase (Nakarai Chemical, Kyoto).

Results

Increased Expression of Neu3 in Human Colon Cancers. To assess Neu3 expression levels, we performed quantitative RT-PCR assays with competitor cDNA. Fig. 1 shows representative results of experiments for Neu3 expression in matched tumor and adjacent nontumor mucosa. Marked increase of Neu3 mRNA was detected in all of the cases for tumor as compared with nontumor tissue. Results of representative quantitative RT-PCR for 10 of 32 cases are shown in Fig. 1*a* and relative levels for 20 of 32 cases in Fig. 1*b*. A 3- to 100–fold increase in the amount of mRNA in colon cancers was evident after normalization for the cDNA of the β-actin gene as an internal control. The sialidase activity was then measured with ganglioside as the substrate by using the particulate fraction of the tissue homogenates (Fig. 1*c*). Activity assays of 50 paired tumor tissues and adjacent nontumorous tissues revealed a statistically significant increase in sialidase activity in the tumor tissues, although the ratio was not always as high as for the mRNA level. There was no significant difference in activity and mRNA levels with different intestinal locations and pathological stages. Fetal colon showed higher Neu3 mRNA levels than nontumor tissues (Fig. 1*a Right*). *In situ* hybridization analysis with antisense probe demonstrated positive signals to be localized to carcinoma cells, not to surrounding stromal cells, with no clear signals with the sense probe (Fig. 2), indicating increased Neu3 expression in cancer cells themselves.

Down-Regulation of Neu3 in Apoptosis-Induced Human Colon Cancer Cells. To cast light on the molecular mechanisms underlying the increased Neu3 expression in colon cancer, we examined Neu3 changes in colon cell lines during differentiation and apoptosis induced by sodium butyrate (NaBT) treatment (23–26). During the culture, we recognized morphological changes including cell enlargement and flattening after 2 days of treatment and detachment and floating in the medium after 72 h (data not shown), as described (26). The treatment induced differentiation and apoptosis in the human colon carcinoma HCT-15, DLD-1, and Colo205 cell lines, as assessed by alkaline phosphatase activity assays and staining with annexin V, respectively. Fig. 3*a* shows representative results for HCT-15 and DLD-1 cells. Alkaline phosphatase activity, a marker of cell differentiation (27), was increased 35- to 105-fold in these cell lines by NaBT treatment. Flowcytometry analysis with annexin V, a marker of early-stage apoptosis, showed the cells to be undergoing apoptosis to some extent with the treatment. The HCT-15 cells demonstrated 17% apoptotic cells with 44% necrotic cells after 50 h of NaBT treatment, whereas the DLD-1 cells had fewer apoptotic (13%) and necrotic (4%) cells (Fig. 3*b*). Under the same conditions we then determined levels of mRNAs and activities for Neu3 and Neu1 sialidases (Fig. 3*c*), both of which are major sialidases in human cells. Competitive RT-PCR analysis showed marked decrease in Neu3 mRNA level, and in contrast, increased Neu1 mRNA after the NaBT treatment, in both cell lines. Activity was measured by using gangliosides and 4-methylumbellyferyl-neuraminic acid as substrates for Neu3 and Neu1, respectively, and shown to parallel mRNA levels for both Neu3 and Neu1. Colo205 cells possessing Neu3 activity comparable to HCT-15 showed 22% apoptotic and 35% necrotic cells with NaBT treatment. It should be noted here that DLD-1 cells having higher Neu3 exhibited less extent of apoptosis than HCT-15 and Colo205 cells with lower Neu3.

Suppression of Apoptosis by Transfection of Neu3 Gene. As the results of NaBT treatment suggested that elevated Neu3 expression may cause suppression of apoptosis, we introduced the Neu3 gene into colon cancer HCT-116 cells, in an ecdyson-based inducible system (28). When the positive transfectant was treated with ponasterone A, an inducer of the ecdyson-inducible promotor, Neu3 sialidase activity was increased 5- to 7-fold whereas the activity in mock transfectants and untreated cells remained at the basal level (Fig. 4*a*). The growth rate of the cells did not show any significant difference even after addition of ponasterone A (data not shown). When positive transfectants were treated with NaBT, they underwent apoptosis (42%) to a large extent before addition of ponasterone A but not as markedly (7%) with the inducer, as determined by annexin V detection (Fig. 4*b*). Because NaBT treatment has been reported not to bring about full differentiation of HCT-116 cells (29), the cells were further treated with brefeldin A to induce terminal differentiation and apoptosis. Like NaBT treatment, brefeldin A decreased the sialidase activity by 40–55% in the cells without ponasterone, but much less change (5–12%) with the reagent. Analysis of the brefeldin A-treated cells by terminal deoxynucleotidyltransferase-mediated dUTP end labeling revealed significant differences in apoptosis between Neu3 transfectants with and without ponasterone A (Fig. 4*c*). As with NaBT treatment,

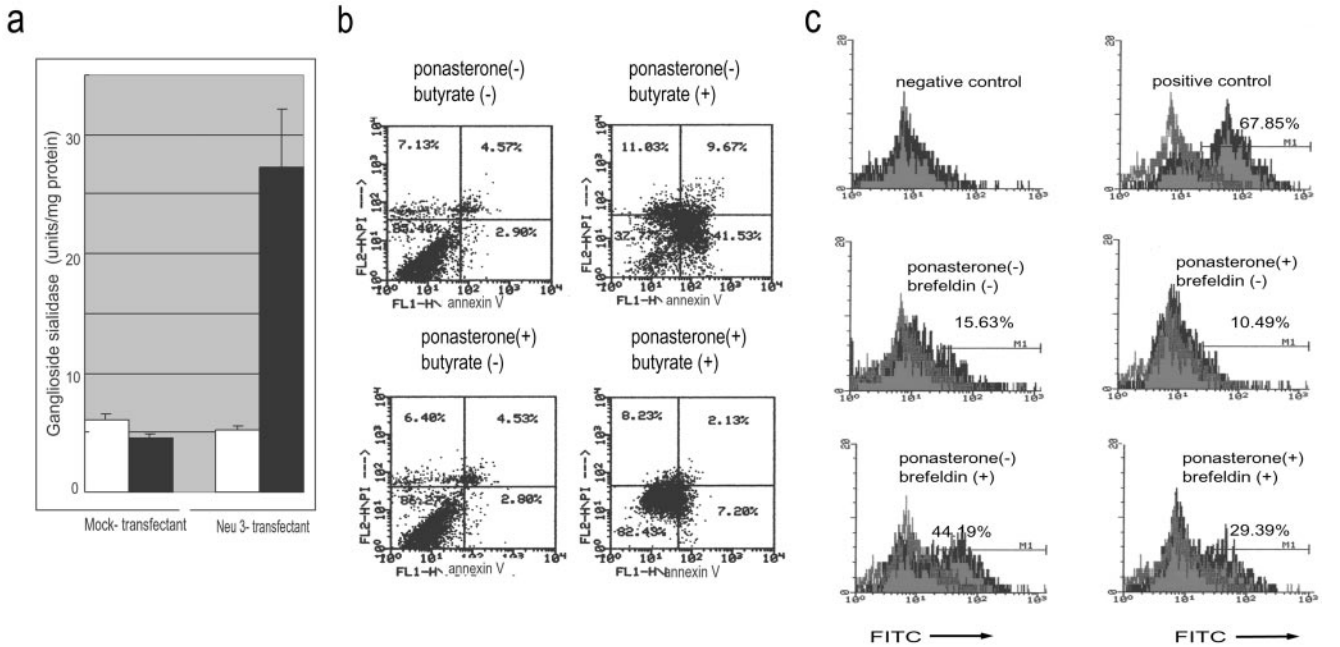

Fig. 4. Apoptosis in Neu3 overexpressing human colon cancer cells. Ganglioside sialidase activity was induced by ponasterone A (closed columns) for 24 h in Neu3-positive transfectants (*a*) as described in *Materials and Methods*. Suppression of apoptosis was detected with annexin V staining (*b*) and terminal deoxynucleotidyltransferase-mediated dUTP end labeling (*c*) in ponasterone-treated Neu3-positive transfectants 48 h after treatment with NaBT (5 mM) and brefeldin A (0.1 μg/ml), respectively. Negative and positive controls in *c* indicate the cells treated with buffer only and DNase (10 μg/ml), respectively.

apoptotic cells were significantly fewer with Neu3-induced than noninduced cells, which was reproducible in three experiments even though the difference was not remarkable (10–15%), indicating suppression of apoptosis by up-regulated Neu3.

Bcl-2 Induction and Caspase Suppression by Increased Neu3 Expression. As Thompson *et al.* (30) reported, NaBT treatment of human colon cancer cells decreases expression of the antiapoptotic protein Bcl-2. We examined Bcl-2 protein levels by Western blots of the colon cancer cell homogenates. As predicted, the Bcl-2 level in HCT-15 cells was decreased after NaBT treatment, under conditions where Neu3 was down-regulated (Fig. 3*c*). In contrast, transient transfection of Neu3 into HCT-15 cells led to a significant increase in Bcl-2 level and scarcely decreased even with NaBT (Fig. 5*a*). In DLD-1 cells Bcl-2 protein was not detectable in any condition tested; probably other Bcl-2 family members such as Bcl-XL participated as antiapoptotic proteins instead as reported (31). Like Neu3-transfected HCT-15 cells, stable transfectants of HCT-116 cells showed increased Bcl-2 protein levels after Neu3 induction (Fig. 5*b*). We further evaluated caspase-3 and –9 expression by the cleavage products with antibody specific for the fragments. Consistent with the results on Bcl-2 above, the cleavage level of procaspase-3 was found to be higher in NaBT-treated cells compared with control, but Neu3 transfection lowered the cleavage activity (Fig. 5*c*). With regard to caspase-9, similar, but smaller, changes were observed (data not shown). Furthermore, DNA macroarray analysis identified several apoptosis-associated genes including RB-2 (GenBank accession no. X74594) and TRAIL receptor (GenBank accession no. U90875) to be down-regulated in Neu3-induced HCT-116 cells. Semiquantitative RT-PCR followed by densitometric analysis confirmed that the expression ratios to β-actin were 0.42 and 0.71 for RB-2 and TRAIL receptor, respectively.

Apoptosis Suppression by Neu3 Reaction Product. To verify a possible involvement of Neu3 reaction products in apoptosis, we observed alteration of the ganglioside pattern by TLC after separation on a

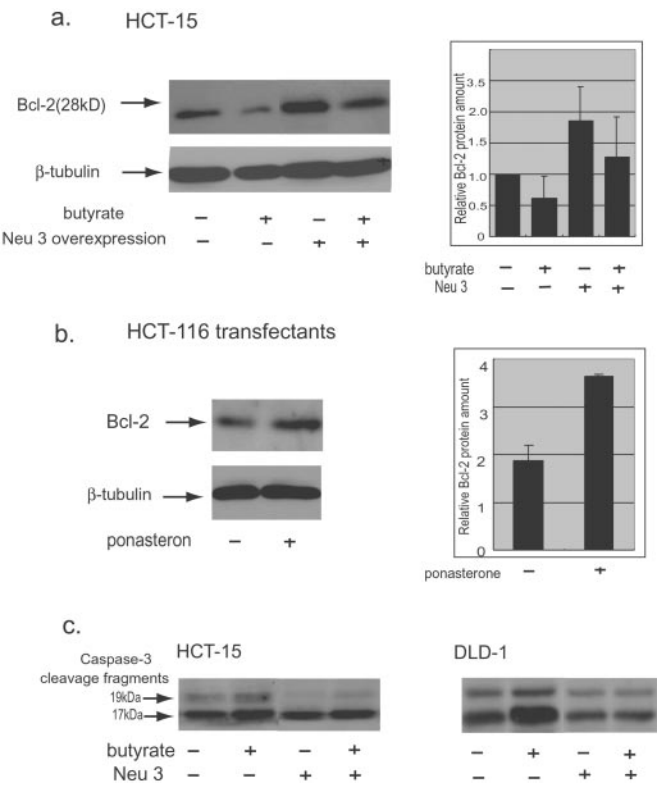

Fig. 5. Alteration of Bcl-2 and caspase-3 expression level by Neu3 overexpression. The levels of Bcl-2 protein (*a* and *b*) and cleavage of procaspase-3 (*c*) were assessed by Western blotting in Neu3-transient transfectants and NaBT-treated cells (HCT-15 and DLD-1) and Neu3–induced HCT-116 cells. Representative photographs are shown, and quantitative data for Bcl-2 levels are presented from three experiments (*a* and *b Right*). β-Tubulin was determined as internal control.

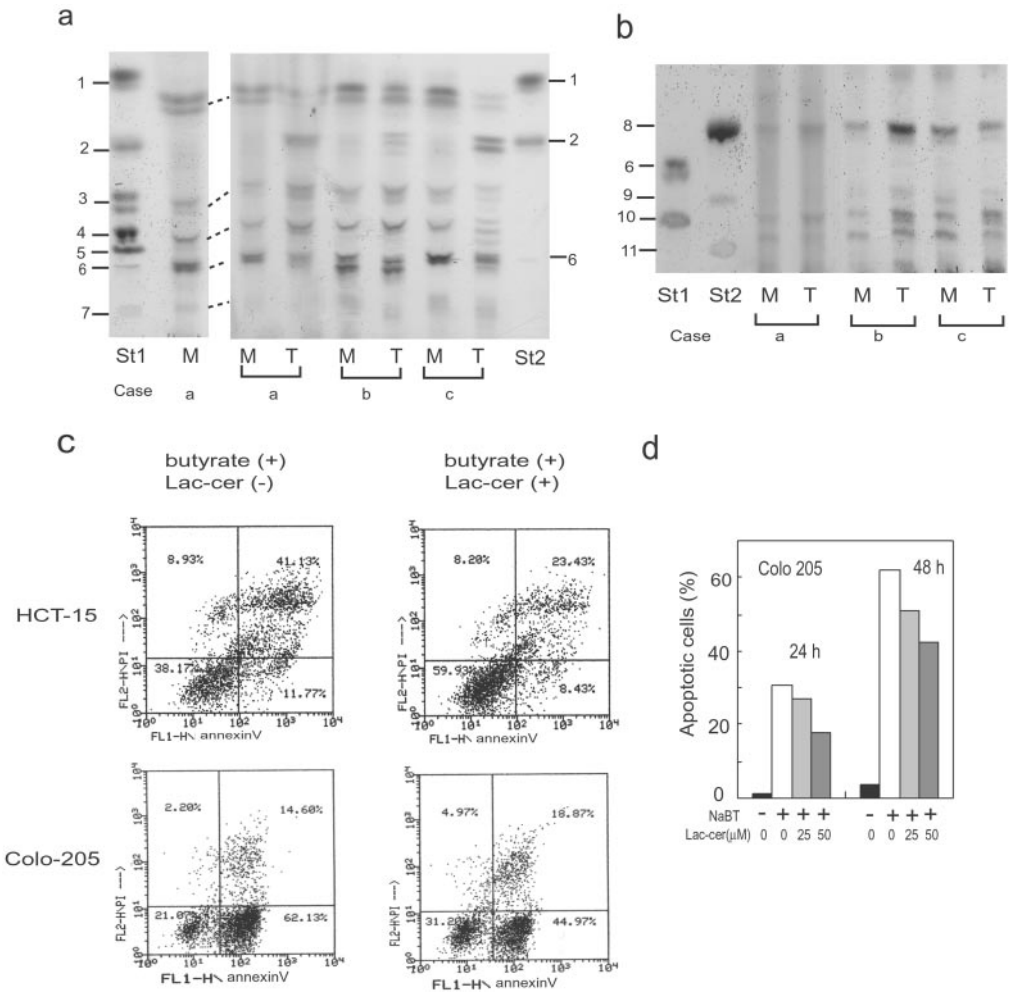

Fig. 6. Accumulation of Lac-cer in colon cancers and its effect on apoptosis. Glycolipids extracted from colon cancers and adjacent noncancerous mucosa were analyzed by TLC as described in *Materials and Methods*, and three representative patterns of five cases tested are shown. Neutral glycolipid (*a*) and ganglioside (*b*) fractions were developed in chloroform/methanol/water (60:40:9, vol/vol/vol) containing 0.02% $CaCl_2$ and in chloroform/methanol/water (60:35:8, vol/vol/vol), respectively. Two different runs (*a*) are shown with dotted lines indicating equivalent bands. Glycolipids were visualized with orcinol-H_2SO_4. Standard glycolipids used were: 1, glucosylceramide; 2, Lac-cer; 3, globotriaosylceramide; 4, globoside; 5, neolactotetraosylceramide; 6, Le^x; 7, Le^a; 8, GM3; 9, α2-3 sialyl neolactotetraosyl ceramide; 10, sialyl-Le^x; 11, sialyl-Le^a. M, mucosa; T, tumor. (*c*) Effect of Lac-cer on apoptosis was examined by annexin V staining in NaBT-treated colon cancer cells. Lac-cer (25 and 50 μM) was added to the culture, and the percentage of apoptotic cells (*d*) was analyzed after 24 h and 48 h of NaBT treatment.

DEAE-Sephadex A25 column. TLC patterns of neutral and acidic glycolipid fractions are shown in Fig. 6 *a* and *b*, respectively. Doublet bands in the chromatogram seem to be caused by differences in the ceramide part of the glycolipids. The neutral component with slightly slower mobility than glucosylceramide probably corresponds to galactosylceramide (Gal-cer), because of its sensitivity to β-galactosidase. Thus neutral glycolipids were composed of five major components with similar mobilities to Gal-cer, lactosylceramide (Lac-cer), globotriaosylceramide, globoside, and Le^x in agreement with previous observations (32, 33). Ganglioside fractions contained three major components, including the two with similar TLC mobilities to GM3 and sialyl-Le^x. The third band migrating between sialyl-Le^x and sialyl-Le^a could be sialyl α2–6 neolactotetraosyl ceramide, as predicted based on previous reports (34, 35). Detailed analyses of these glycolipids were not performed because of a limited amount of sample. If their chromatographic behavior can indicate that they correspond to their respective standard glycolipids having similar mobilities, changes most characteristic of colon cancer were a marked increase of Lac-cer and a decrease of Gal-cer in five cases tested (three typical cases in Fig. 6) as compared with nontumor mucosa. In addition, sialyl-Le^x tended to be increased and Le^x was rather slightly decreased in colon cancer, although the changes were not detected in all of the cases. The Neu3-induced transfectants also showed a slight increase (130% of control) of Lac-cer but no significant change in other glycolipids, when compared with the cells without ponasterone A. These data indicate Lac-cer probably is one of the product molecules of elevated Neu3 in colon cancer. We then examined the effect of Lac-cer on apoptosis of the colon cancer cells by annexin V detection after NaBT treatment (Fig. 6*c*). Addition of Lac-cer to the culture led to a decreased percentage of apoptotic cells in a time- and dose-dependent manner (Fig. 6*d*), suggesting apoptosis suppression by Lac-cer in the cancer cells.

Discussion

The present study demonstrated that Neu3 expression is increased in colon cancer in terms of both mRNA level and enzyme activity. *In situ* hybridization further revealed Neu3 to be highly expressed in epithelial tumor cells. Down-regulation was evident with NaBT-induced differentiation and apoptosis of the cancer cells whereas Neu3 overexpression resulted in inhibition of apoptosis accompanied by increased Bcl-2 and decreased caspase expression. The difference in the ratios of increase between mRNA and activity probably is caused by an involvement of posttranscriptional modifications such as phosphorylation of the sialidase protein (A. Sasaki, K. Hata, and T.M., unpublished work). In addition, endogenous content of gangliosides present in the enzyme preparation may cause the difference in mRNA and activity, because tumor tissues tended to have lower content of gangliosides cleavable by Neu3. In fact, activity was increased further with additional gangliosides as substrate, despite the K_m value similar to that of mucosa enzyme (data not shown). The results for NaBT treatment provide an important clue as to the biological significance of Neu3 expression in colon cancer, the resultant decrease being associated with differentiation and apoptosis. The opposing expression patterns of Neu3

and Neu1 may reflect different roles, the former playing a major role in ganglioside modulation because of its specificity, and the latter likely to be responsible mainly for glycoprotein degradation. A low Neu1 level in undifferentiated cells, therefore possibly causing hypersialylation of the carbohydrate portion of glycoproteins, is in line with the observation that malignant cells are often hypersialylated (6, 36). We previously observed decreased Neu1 activity in transformed rat 3Y1 fibroblasts (37) and suppression of malignant phenotype of B16 melanoma cells by Neu1 overexpression (22), although one controversial report (38) of increased sialidase activity in human colon tumor probably reflected change in the same Neu1 enzyme, because of the use of 4-methylumbellyferyl-neuraminic acid as substrate. Decreased Neu3 in differentiated cells, on the other hand, would lead to reduction in ganglioside degradation, consistent with previous reports presenting evidence for an increase of GM3 synthase during NaBT treatment (23). Elevated Neu3 expression in undifferentiated cells in the fetal colon indicates a close relation to cell differentiation during development. Although the present results seem to contradict the previous observations of increased expression of Neu3 during differentiation of human (39, 40) and mouse (21) neuroblastoma cells, the enzyme might be expected to behave in a different manner in cell origin. As apoptosis is recognized as part of the terminal differentiation process in the colonic crypt, it is feasible that its suppression occurs in Neu3 overexpressing cells with increase in Bcl-2 expression. This finding would indicate that Neu3-associated apoptosis occurs, at least partly, through the mitochondrial pathway.

In view of the unique character of Neu3 in subcellular localization and strict substrate specificity, ganglioside modulation by Neu3 would be expected to trigger signaling pathways at the cell surface. The increased Lac-cer in colon cancer, consistent with the previous report (32), may be one of the results of Neu3 enzyme reactions, probably exerting an influence on apoptosis as a negative regulator. It is also of interest that colon cancer showing a tendency to decrease Le^x may be related to apoptosis suppression, as Akamatsu *et al.* (41) reported that increased Le^x is involved in the process of apoptosis in HT-29 colon cancer cells. Sialyl-Le^x, on the other hand, tended to be elevated in the cancer tissues, as generally accepted, even under high Neu3 expression. However, it is not contradictory, because our preliminary experiments showed the lipid to be a poor substrate for Neu3. In line with previous observations that the level of GD3 synthesis elevates in apoptotic cells (42, 43), GD3 could be hydrolyzed rapidly by increased Neu3 in colon cancer cells, possibly leading to suppression of apoptosis, although a significant change in GD3 was not detectable in our case. Inhibitory role of Neu3 in apoptosis could be mediated by accumulation of the reaction product such as Lac-cer or by rapid degradation of GD3. Through modulation of gangliosides Neu3 may influence not only the mitochondrial apoptotic pathway but also the apoptosis-related molecules at the death receptors' level, as described (44, 45), that Neu3 is within microdomain, rafts, at the cell surface. Taken together, the results suggest that increased Neu3 might cause disturbance of apoptosis signaling through ganglioside modulation. Neu3 may represent an effective molecular marker for colon cancer, providing a target for diagnosis and treatment.

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